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Sensitivity and Specificity of Serologic Assays for Detection of Human Infection with 2009 Pandemic H1N1 Virus in U.S. Populations▽

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Swine origin 2009 H1N1 influenza virus has spread globally to cause the first influenza pandemic of the 21st century. Serological studies can improve our understanding of the extent of human infection and risk factors associated with the transmission of this pandemic virus. The "gold standard" for serodiagnosis of human influenza virus infection is the detection of seroconversion between acute- and convalescent-stage samples. However, the timing of seroepidemiological investigations often precludes the collection of truly acute-phase sera, requiring development of serological criteria for evaluating convalescent-phase sera that optimize detection of true positives and true negatives. To guide seroepidemiological investigations into the spread of the novel 2009 pandemic H1N1 virus, we characterized serum antibody responses to 2009 H1N1 virus in 87 individuals with confirmed viral infection and 227 nonexposed U.S. individuals using microneutralization (MN) and hemagglutination inhibition (HI) assays. Sensitivity and specificity were determined for each assay alone and in combination for detection of 2009 H1N1 virus-specific antibodies in convalescent-phase sera. Although the HI assay was more specific for detecting antibody to 2009 H1N1, the MN assay was more sensitive, particularly for detecting low-titer seroconversions. A combination of titers (MN ≥ 40 and HI ≥ 20) provided the highest sensitivity (90%) and specificity (96%) for individuals aged <60 years and 92% specificity for adults aged ≥60 years for detection of serologically confirmed 2009 H1N1 infections in U.S. populations during the first pandemic waves. These studies provide an approach to optimize timely serological investigations for future pandemics or outbreaks of novel influenza viruses among humans.

Since first emerging among humans in North America in the spring of 2009, the swine origin 2009 H1N1 influenza virus has spread globally to cause the first influenza pandemic in over 40 years (2, 8, 28). Estimating the total number of pandemic H1N1 (2009 H1N1) virus-infected persons is challenging, since estimates based on virological laboratory confirmation, and even disease surveillance, vastly underestimate the true number of infected persons (7, 20, 21). Serological studies can provide a better understanding of the extent of human infection with 2009 H1N1 virus in different settings (1, 19). In particular, seroepidemiological studies can assess risk factors for infection and rates of transmission in defined populations by linking detection of serum antibody responses as retrospective evidence of infection with information on illness, demographics, and behavioral factors. Because such studies focus on confirmation of infections at the individual level, rather than infection rates within a population, they require development of serological criteria that optimize detection of true positives and true negatives.

Although detection of seroconversion, a 4-fold or greater rise in the influenza virus antibody titer, between acute- and convalescent-phase sera remains the optimal serodiagnostic approach, the timing of serological investigations often precludes the collection of baseline sera over which to detect seroconversion. In such situations, it may be possible to develop criteria for seropositivity based on a single convalescent-phase serum, if the virus hemagglutinin (HA) is sufficiently novel with respect to seasonal influenza viruses. The 2009 H1N1 virus is antigenically and genetically distinct from seasonal H1N1 viruses that have circulated in the last 60 years (11). Nevertheless, studies in Europe and the United States demonstrated that, prior to the 2009 pandemic, approximately 20 to 30% of adults 60 to 65 years old and older possessed serum antibody cross-reactive with 2009 H1N1 virus (13, 20, 22).

The hemagglutination inhibition (HI) assay has long been used to detect serological responses to influenza virus infection or vaccination. An HI titer of ≥40 is associated with a 50% or greater reduction in the risk of influenza virus infection or disease in susceptible populations (9, 14). More recently, virus

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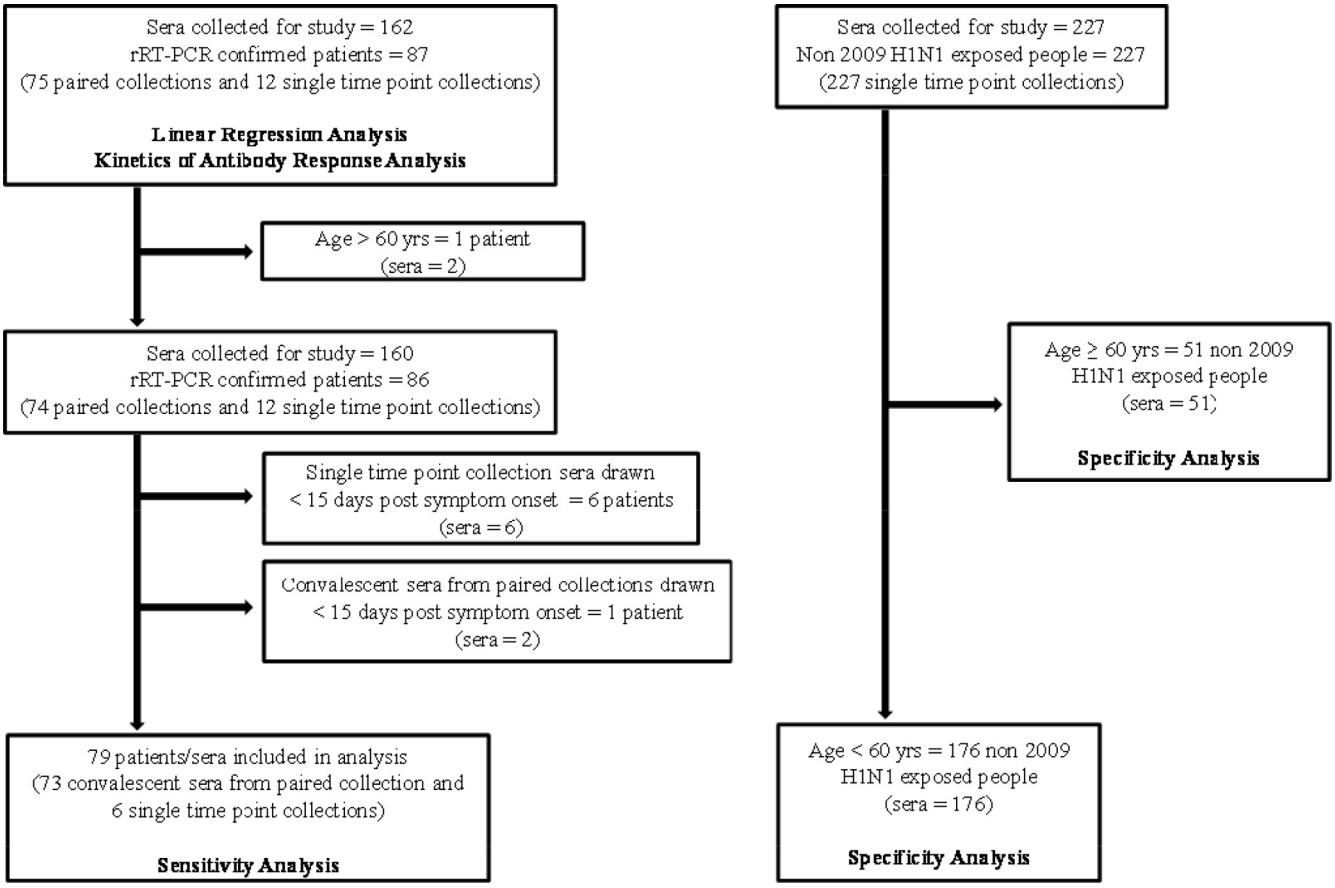

FIG. 1. Sera included and excluded in analyses.

neutralization or microneutralization (MN) assays have also been used, because they detect functional neutralizing antibodies and, in some cases, offer greater sensitivity than traditional HI assays for the detection of antibodies following influenza virus infection or vaccination, particularly with novel influenza A viruses, such as avian-origin H5N1 viruses (12, 24). However, there are only limited data on the comparative sensitivities of these serological assays for the diagnosis of infection with 2009 H1N1 virus (4, 6). In this study, we aimed to bridge this knowledge gap and describe an approach to compare the relative sensitivities and specificities of the MN and HI assays against a novel virus, such as 2009 H1N1. We establish seropositivity criteria for convalescent-phase sera to identify 2009 H1N1 influenza virus-infected persons and discuss the relative values of the assays for different serological purposes.

MATERIALS AND METHODS

Serum collection. Sera (n = 162) were collected from 87 U.S. residents (aged 3 months to 80 years) with 2009 H1N1 virus infection confirmed by real-time reverse transcription PCR (rRT-PCR) from April to August 2009. The proportions of confirmed cases by age group were as follows: <10 years, 8%; 10 to 19 years, 30%; 20 to 29 years, 48%; 30 to 39 years, 8%; 40 to 59 years, 5%; >60 years, 1%. These included sera collected at a single time point (n = 12; 7 to 23 days post-symptom onset [p.s.o.]) or paired sera from 75 individuals collected 1 to 77 days p.s.o. (Fig. 1). A total of 227 sera from U.S. residents aged 6 months to 88 years were used for the specificity analysis; 168 sera were collected prior to the circulation of the 2009 H1N1 virus, as previously described (13), and 59 sera were from individuals with rRT-PCR-confirmed seasonal H1N1 virus infection in the 2008–2009 season collected 3 to 6 months p.s.o. (Fig. 1). The collection and testing of serum samples at CDC was considered to be a public health, nonresearch activity that was exempt from human subject review.

Serological procedures. For the HI assay, sera were first treated with receptor-destroying enzyme (RDE) (Denke-Seiken, Japan), followed by heat inactivation at 56°C for 30 min. Sera containing nonspecific agglutinins were preadsorbed with turkey erythrocytes. For the MN assay, sera were first heat inactivated at 56°C for 30 min. The sera were tested by HI assay using 0.5% turkey erythrocytes and by MN assay according to previously published procedures and using A/Mexico/4108/2009, an A/California/7/2009-like 2009 H1N1 virus, which was propagated in 10- to 11-day-old embryonated chicken eggs (13, 18, 24). For both assays, serial 2-fold dilutions of serum (1:10 to 1:1,280) were tested in duplicate. HI or MN titers were expressed as the reciprocal of the highest dilution of serum that gave complete hemagglutination or 50% neutralization, respectively.

Linear regression model and statistical analyses. Linear regression models using antibody titers from all rRT-PCR-confirmed 2009 H1N1 sera (Fig. 1) were performed to estimate the correlation between serum antibody titers measured by MN and HI assays and to determine the predicted 2009 H1N1 virus MN titers corresponding to 2009 H1N1 virus HI titers. HI titers (ranging from 5 to 640) and MN titers were transformed to $\log_2$. To best determine the relationship between HI and MN titers and to observe the proportion of variation in the MN titers that can be explained by HI titers, the following independent variables were included in the model: $\log_2$ HI titer, $\log_2$ HI titer^2, and $\log_2$ HI titer^3. An age variable was not included in the model, as the majority of the rRT-PCR-confirmed sampled population had ages that ranged between 10 and 29 years. Using backward elimination, only variables that were statistically significant ($P \leq 0.05$) were kept in the model.

The percent sensitivity and specificity achieved at different individual and combination titer cutoffs were determined using only sera collected 15 or more days p.s.o. (Fig. 1) from rRT-PCR-confirmed 2009 H1N1 cases (n = 79) and sera belonging to nonexposed populations (n = 227). The significance of geometric mean titers and seroconversion rates was determined by analysis of variance

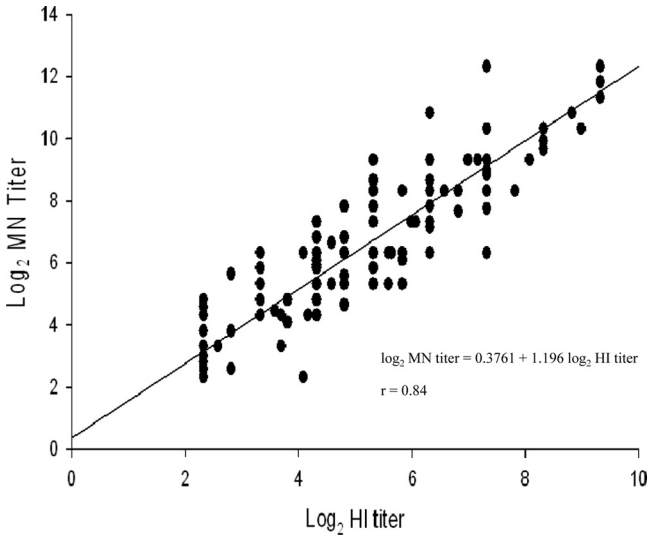

FIG. 2. Linear regression and correlation between $\log_2$ MN titers and $\log_2$ HI titers.

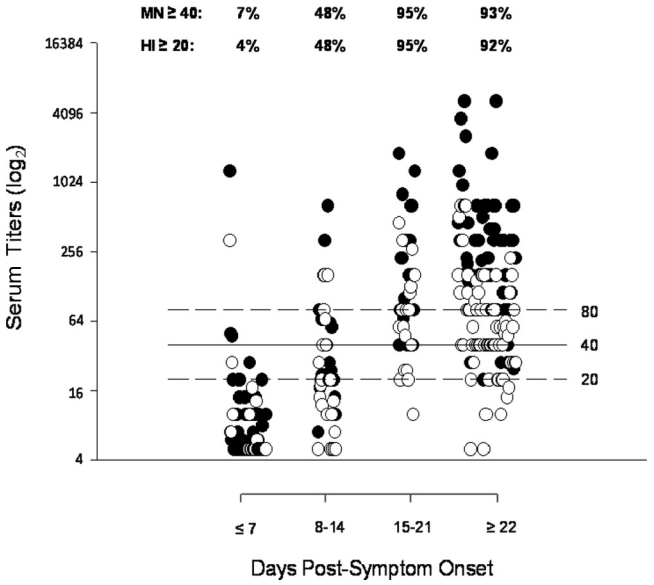

FIG. 3. Kinetics of antibody response in rRT-PCR-confirmed pandemic H1N1 cases. HI and MN titers are grouped by number of days post-symptom onset based on 7-day intervals for 162 serum samples from 87 cases. The number of serum samples collected per 7-day interval post-symptom onset were as follows: ≤7 days, 46 sera; 8 to 14 days, 23 sera; 15 to 21 days, 21 sera; ≥22 days, 72 sera. Individual MN (●) and HI (○) titers, as well as the proportion of individuals who achieved an MN titer of ≥40 or the corresponding HI titer of ≥20, are shown. The lines indicate titers of 20, 40, and 80.

(ANOVA) and chi-square tests, respectively. *P* values of less than 0.05 were considered significant. SAS V9.1 software (SAS Institute Inc., Cary, NC) was used for all statistical analysis.

RESULTS

Correlation and linear regression analysis of serological tests. Sera from 87 confirmed cases were tested by MN and HI assays for the presence of antibody against 2009 H1N1 influenza virus (Fig. 2). A strong positive correlation (Spearman's rank correlation, $r = 0.84$) was noted between HI and MN titers. The final linear regression model, which included only statistically significant variables ($P \leq 0.05$), was as follows: $\log_2$ MN titer = $0.3761 + 1.196 \log_2$ HI titer. The assumptions of linearity and homogeneity of variance were met, as residual plots showed neither a linear nor an outward/inward curvature pattern (data not shown). As shown in Table 1, the nearest predicted discrete MN titer was generally 2-fold higher for HI titers of ≤160 and 4-fold higher for HI titers of 320 and 640.

Kinetics of antibody response analysis. Figure 3 shows the kinetics of antibody response in all rRT-PCR-confirmed cases grouped at 7-day-interval time points to depict the rising titer trend over time. The proportion of individuals with HI titers of ≥40, a titer threshold generally associated with a 50% reduction in the risk of influenza illness in susceptible populations (14), increased from 2% to 26% after the first week of infection and rose to 71 and 76% for sera collected between 15 and 21 or ≥22 days p.s.o., respectively. We also assessed the proportion of individuals who achieved an MN titer of ≥40 (corresponding to an HI titer of ≥20, as predicted by our linear regression model). Only 48% of individuals whose sera were collected between 8 and 14 days p.s.o. achieved these titers, whereas greater than 90% of individuals whose sera were collected 15 or more days p.s.o. achieved them. As expected, the difference between the geometric mean titers for sera collected less than 15 days p.s.o. (MN = 15; HI = 9; $n = 69$) and sera collected during the convalescent phase, or ≥15 days p.s.o. (MN = 203; HI = 61; $n = 93$), was statistically significant for both assays ($P < 0.01$). Nevertheless, there was no significant difference between the geometric mean titers of sera collected 15 to 21 days p.s.o. (MN = 160; HI = 66; $n = 21$), sera collected 22 to 28 days p.s.o. (MN = 247; HI = 59; $n = 50$), and sera collected >28 days p.s.o. (MN = 163; HI = 61; $n = 22$). Among 55 individuals for whom well-timed paired sera were available, seroconversions were detected more often by MN (91%) than by HI (84%) assay. Furthermore, seroconversion detections were statistically higher ($P < 0.05$) by the MN than by the HI assay, when data were stratified to include only paired sera with acute-phase sample titers of 20 or less by both assays. Among these data, the MN assay detected 100% (41/41) of the seroconversions, while the HI assay detected only 85% (35/41).

TABLE 1. Predicted 2009 H1N1 MN titers corresponding to 2009 H1N1 HI titers using a linear regression model

HI titer	Predicted MN titer	Discrete predicted MN titer	*n*
5	9	5	43
10	20	20	7
20	47	40	13
40	107	80	19
80	245	160	15
160	560	320	11
320	1,282	1,280	3
640	2,932	2,560	4

Sensitivities and specificities of 2009 H1N1-specific serological tests. To establish serological criteria for detection of 2009 H1N1 virus infection in convalescent-phase sera, we per-

TABLE 2. MN and HI assay sensitivity and specificity summary

Parameter and group	*n*	Value [% (95% CI[a])]						
		MN assay with cutoff:		HI assay with cutoff:		MN titer, ≥40; HI titer, ≥20	MN titer, ≥40; HI titer, ≥40	MN titer, ≥80; HI titer, ≥40
		≥40	≥80	≥20	≥40			
Sensitivity								
Confirmed cases, ages <60 yr	79	94 (85–98)	80 (69–88)	92 (84–97)	75 (63–84)	90 (81–95)	75 (63–84)	68 (57–78)
Specificity								
All nonexposed individuals								
Ages <60 yr	176	83 (76–88)	94 (89–97)	91 (86–95)	97 (93–99)	96 (92–98)	98 (95–100)	99 (96–100)
Ages 0–39 yr	127	91 (85–95)	96 (91–99)	91 (84–95)	96 (91–99)	97 (92–99)	98 (93–99)	98 (94–100)
Ages 40–59 yr	49	61 (46–74)	88 (75–95)	94 (82–98)	100 (91–100)	94 (82–98)	100 (91–100)	100 (91–100)
Ages ≥60 yr	51	59 (44–72)	84 (71–93)	92 (80–97)	94 (83–98)	92 (80–97)	94 (83–98)	94 (83–98)

[a] CI, confidence interval.

formed a sensitivity (using sera from 79 rRT-PCR-confirmed cases <60 years of age collected 15 days or more p.s.o. [Fig. 1]) and specificity (using sera from a nonexposed population) analyses for the MN and HI assays. As shown in Table 2, the titer cutoff value that provided the highest sensitivity was an MN titer of ≥40 (94%) or an HI titer of ≥20 (92%). Since the majority (86%) of our rRT-PCR-confirmed cases were <30 years of age, it was not possible to stratify the sensitivity results by age or to estimate sensitivity in adults ≥60 years of age.

Because prepandemic cross-reactive antibody to the 2009 H1N1 virus has been demonstrated particularly in older adults (13, 16, 17, 20, 22), we compared the specificities for detection of 2009 H1N1 virus antibody in different age groups. For individuals <60 years of age, the MN titer cutoff that gave optimal sensitivity (≥40) was only 83% specific, whereas the comparable HI titer cutoff (≥20) gave 91% specificity. The reduced specificity of the MN assay among these individuals was primarily due to the lower specificity (61%) observed in adults 40 to 59 years of age. The MN assay was also less specific than the HI assay among adults ≥60 years of age; the specificity of an MN titer of ≥40 or an HI titer of ≥20 was 59% or 92%, respectively. Overall, for those <60 years of age, a 2-fold increase in the cutoff titer (MN titer of ≥80 or HI titer of ≥40) considerably improved the specificity but substantially reduced the sensitivity to unacceptable levels (80 or 75%, respectively).

We next assessed whether a combination of MN and HI titers could maximize sensitivity and specificity (Table 2). Combining an MN titer of ≥40 and an HI titer of ≥20 resulted in a sensitivity of 90% and a specificity of 96% for all ages of <60 years and a specificity of 92% for the ≥60-year age group. Although combinations using higher titer cutoffs modestly improved specificity, the sensitivity dropped to ≤75%. These results suggest that seropositivity criteria based on a combination of serological titers can provide maximal sensitivity and specificity for the detection of 2009 H1N1 virus-specific antibody in individuals <60 years of age and specificity comparable to that of the HI assay alone in those ≥60 years of age.

DISCUSSION

The 2009 pandemic has highlighted the need for timely studies to investigate the extent of age-specific human infection after multiple pandemic waves in different geographic regions (19). Such studies can estimate total numbers of infections upon which to base more accurate estimates of rates of severe or fatal disease. They may also provide policy makers with a better understanding of the proportion of susceptible persons remaining in populations in order to better predict the public health impact of successive pandemic waves. To better understand the relative benefits of the HI versus the MN assay for the detection of 2009 H1N1 virus-infected persons, we characterized serum antibody responses to 2009 H1N1 virus in rRT-PCR-confirmed cases using both serological assays and confirmed that the titers obtained by either assay were highly correlated. Furthermore, using an additional set of sera from non-2009 H1N1-exposed populations, we assessed the relative sensitivities and specificities of both assays. Although the HI assay alone was more specific for detecting antibody to 2009 H1N1 virus, the MN assay was significantly more sensitive for detecting low-titer seroconversions. Finally, based on our sensitivity and specificity analyses, we identified a combination of threshold titers (MN, ≥40, and HI, ≥20) that provided the highest sensitivity and specificity to identify 2009 H1N1 virus-infected persons <60 years of age and high specificity for adults aged ≥60 years using only convalescent-phase sera and in the absence of demonstrable seroconversion in paired sera. These criteria were used to facilitate the analyses of multiple seroepidemiological investigations conducted in the United States during the first wave of the 2009 pandemic.

The kinetics of antibody responses in rRT-PCR-confirmed 2009 H1N1 cases confirmed that the optimal timing of acute-phase serum collection is within 1 week of symptom onset. Although 90% of cases achieved threshold titers for seropositivity (MN titer of ≥40 and HI titer of ≥20) by day 15 p.s.o., sera collected 22 to 28 days p.s.o. had the highest geometric mean titers, suggesting that this remains the optimal time frame for the collection of convalescent-phase sera. These results are consistent with those of Miller et al. (20) and Hung et al. (15), who reported that 11% of confirmed 2009 H1N1 cases in England failed to develop HI antibody titers of ≥32 and 10% of confirmed cases in Hong Kong failed to develop neutralizing antibody titers of ≥40. Similar to our results, Hung et al. (15) also found that the MN assay detected a higher seroconversion rate (89%) than the HI assay (82%).

The HI assay detects antibodies that bind near the receptor binding site of the viral HA, blocking the interaction of HA with sialic acid receptors on erythrocytes and inhibiting their

agglutination. Virus neutralization assays, such as the MN assay, detect antibodies that neutralize the virus by inhibiting viral entry and/or replication in mammalian cells, including antibodies recognizing epitopes within the stem region of HA that block membrane fusion and that are conserved among viruses of different influenza A virus subtypes (26). Detection of cross-reactive antibodies to the stem region could consequently lower the specificity of the MN assay, particularly in the adult and older adult populations, who presumably have had greater exposure to different influenza A viruses throughout their lifetimes.

Several studies have used an HI titer of ≥40 as a marker of infection with or immunity to 2009 H1N1 virus (20, 23). This is a reasonable approach for large-volume seroprevalence and seroincidence studies and for optimal rapidity of reporting results following successive pandemic waves. However, our data suggest that this titer threshold may underestimate the numbers of 2009 H1N1 virus-infected individuals. On the other hand, due to its lower specificity in adults aged 40 and over, the use of the MN assay alone may overestimate 2009 H1N1 virus infections in the age group for U.S. populations. Where resources permit, and particularly when studies seek to identify 2009 H1N1 virus-infected individuals rather than population rates, our results suggest that the use of both assays and the combination titer achievements provide optimal sensitivity and specificity. However, it should be noted that while it provides a sensitive and specific serological marker for infection, the combination of titer achievements cannot be correlated with a level of protection against the pandemic virus. Furthermore, once a pandemic virus becomes seasonal, as is now the case for the 2009 H1N1 virus (10), serological confirmation of human infection will once again require detection of seroconversion by either assay, the gold standard for all influenza virus serodiagnosis.

Our study had several limitations. The age distribution of our rRT-PCR-confirmed cases differed from that of 2009 H1N1 cases based on national estimates (21): the 0- to 4-year age group was underrepresented, and the 5- to 24-year age group was overrepresented. In addition, we were unable to estimate the sensitivity of the assays and titer cutoffs for adults ≥60 years of age. Furthermore, because individuals 80 years old and older exhibit high frequencies of serum antibodies that cross-react with 2009 H1N1 virus, due to structural similarities that exist between the HA molecules of 2009 H1N1 and 1918-like influenza viruses, the seropositivity criteria developed here cannot be applied to this age group (16, 17, 20, 29). Efforts to discriminate preexisting serum cross-reactive antibody from 2009 H1N1 virus infection-induced antibodies in this age group are ongoing in our laboratory.

Studies from China and Singapore found little evidence of preexisting 2009 H1N1 virus cross-reactive antibodies in older adults, even those ≥80 years of age, suggesting that there are geographic or other factors that contribute to the presence of preexisting antibody in human populations (3, 5, 27). These findings support the need for laboratories undertaking seroepidemiological or seroprevalence studies for detection of antibody against novel viruses to individually evaluate the prepandemic age-specific prevalence of cross-reactive antibody in local populations. Furthermore, due to interlaboratory assay variation, caution should be exercised in directly using the seropositivity criteria developed here to identify 2009 H1N1 virus-infected persons based on serological data from other laboratories (25).

In summary, we have demonstrated an approach whereby serological criteria can be developed to identify human infections using only convalescent-phase sera when novel influenza viruses first emerge to infect humans. Assessing the relative sensitivities and specificities of serological assays is an important component in establishing threshold titers used to estimate the extent of seropositivity among populations after the first pandemic waves. We believe these studies may provide a strategy to assist with timely serological investigations for future pandemics or outbreaks of novel influenza viruses among humans.

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We declare that we have no competing financial interests.

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